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PROTECTION OF RAT HEART FROM ISCHAEMIA-REPERFUSION INJURY BY THE 21-AMINOSTEROID U-74389G

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In ischaemia-reperfusion syndromes lipid peroxidation appears an important factor contributing to tissue damage. The 21-aminosteroids (lazaroids) exhibit beneficial effects in various pathological conditions, especially in post-traumatic lesions of the central nervous system, where a peroxidative injury seems to be involved. The aim of our study was to ascertain if one of these compounds, U-74389G, plays a significant role in protecting heart muscle from ischaemia-reperfusion damage. Rat hearts used for heterotopic transplantation represented the experimental model in this investigation.

Animals (Wistar rats weighing 200–250 g) were divided into five groups: controls, untreated and treated donors, untreated and treated recipients. Donors were anaesthetized and heparinized, and the heart was excised through a bilateral thoracotomy, arrested with St Thomas solution and stored in cold saline for 2 hours. For the recipient preparation, a modified Ono's technique was used, and heart reimplantation was performed with a termino-lateral aorto-aortic anasthomosis and a termino-lateral pulmonary-cava anasthomosis. After the anasthomoses were completed hearts were reperfused for 30 min; then hearts were excised and specimens were taken for biochemical and morphological studies. These were conducted on three groups of hearts: (A) hearts reimplanted and reperfused without treatment of the donor or of the recipient animal; (B) hearts subjected to the same procedure but in the presence of U-74389G treatment of donors and recipient rats; (C) control hearts rapidly excised from normal, non-operated animals.

Electron microscopy studies showed, in hearts transplanted without treatment, the typical morphological aspects of lipoperoxidative injury: swollen mitochondria with disrupted cristae, damaged endothelial cells with the nucleous bulging into the lumen and a discontinued endothelial lining with diffuse oedema among the fibers. Lazaroid treatment attenuated most of these damages in hearts of group B.

As for the biochemical findings, the hearts transplanted in the presence of U-74389G treatment had significantly higher ATP and creatine phosphate levels ($P<0.01$) and lower malondialdehyde concentrations ($P<0.05$) with respect to the hearts transplanted without treatment. Furthermore, serum creatine kinase activity was lower in treated than in untreated recipient animals ($P<0.05$).

Taken together, all these results indicate that U-74389G treatment is effective in protecting cardiac muscle from structural and functional ischaemia-reperfusion injuries, at least from those arising during a heart transplantation procedure.

KEY WORDS: ischaemia-reperfusion, heart injury, lazaroids.

INTRODUCTION

Extensive experimental evidence suggests that lipid peroxidation (LPO) and other free radical mediated cytotoxic effects play an important role in the pathogenesis of the injuries induced by ischaemia and reperfusion in various human tissues, among them heart muscle, where this can occur, besides other circumstances, during cardiac surgery or in the case of heart transplantation[1–3]. Not surprisingly, given the number and the complexity of the mechanisms that can account for the formation of free radicals in

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ischaemia-reperfusion conditions, many therapeutic approaches have been proposed to protect human tissues from the injuries due to these reactive chemical species, notably from LPO.

For example, this chain reaction may be inhibited by the use of radical scavengers: cloned human superoxide dismutase proved to be effective in removing the superoxide anion and, consequently, the other reactive oxygen metabolites (ROMs) that may derive from this radical; iron chelators can inhibit the iron catalysed Fenton reaction; also agents that lessen phospholipid mobility (i.e. membrane stabilizers) can limit the propagation of lipid peroxidation.

The 21-aminosteroids (lazaroids) seem to fulfil many of these requirements: their action is many-sided and may be described as follows:

(1) antioxidant effects on cell membranes, owing to their ability to act as free radical scavengers;

(2) stabilizing effect on cell membranes, thus inhibiting the propagation of lipid peroxidation by restricting the movement of peroxyl and alcoxyl radicals within the membrane;

(3) preserved release of endothelium-dependent vascular relaxation factors and protection of the endothelium itself from damage by ROMs and, generally, by free-radicals.

A number of reports indicate that the aminosteroids are of therapeutic benefit in various pathological conditions where an increased free radical formation may be involved. These compounds seem to have a protective effect especially on neuronal viability in ischaemic and post traumatic lesions of the central nervous system[4], while their effectiveness for myocardial salvage after ischaemia and reperfusion is more debated. In fact, from several reports[5–7] it appears that, in various animal models of ischaemia-reperfusion, the pretreatment with lazaroids such as U-74006F and U-74500A can reduce the myocardial injury and enhance the functional recovery. On the other hand, these beneficial effects have not been confirmed by other authors; Ovize *et al.*[8], for example, found that the U-74006F compound, although it appeared as a potent in-vivo inhibitor of lipid peroxidation, failed to limit infarct size in a canine model of myocardial ischaemia and reperfusion.

Given this framework, the aim of the present investigation was to assess the ability of a 21-aminosteroidal compound, U-74389G, in protecting heart muscle from biochemical and morphological alteration induced by ischaemia and reperfusion.

Rat hearts, subjected to heterotopic transplantation, represented the experimental model used in these studies.

MATERIALS AND METHODS

Enzyme, coenzymes and substrates were purchased from Sigma Chemie, Italy. U-74389G was obtained from Upjohn Company, Kalamazoo, MI, USA. All other compounds were of analytical grade.

Animals: study groups and surgical preparation

Wistar rats, weighing 200–250 g, were used in this investigation. Animal treatment was conducted in accordance with the rules prescribed by Italian law in the matter of use and care of laboratory animals. Rats were divided into five groups; controls, untreated and treated donors, untreated and treated recipients. In heart transplantation experiments, donor and recipient rats, fasted from the night before, were anaesthetized with 8% chloral hydrate, given intraperitoneally 0.75 ml/100 g body weight.

Donor preparation. The anesthetized animals were heparinized 3 mg kg^{-1} via the inferior vena cava; after 5 min the chest was entered through a bilateral thoracotomy, the inferior cava divided, pericardium opened, ascending aorta and pulmonary artery dissected free from the surrounding tissues, the heart rapidly excised and arrested by infusion of 5 ml of St Thomas solution directly into the aorta. The inferior vena cava was then ligated and the heart was stored in ice-cold saline solution for 2 h.

Recipient preparation. A modified Ono's[9] technique was used: through a median laparotomy infrarenal aorta and inferior vena cava were exposed by sharp dissection. Lumbar veins, when present, were ligated but not divided. Aorta and cava were dissected free for a length of about 1.5 cm; they were clamped separately and opened with an incision of 7–8 mm. The explanted heart was then reimplanted with a termino-lateral aorto-aortic anastomosis and a termino-lateral pulmonary-cavo anastomosis using a 9 o monofilament continuous suture under microscopic magnification. When the anastomoses were complete, vessel clamps were removed and the heart reperfused. After 30 min of reperfusion hearts were excised and specimens were taken for biochemical and histological studies. These were performed in three groups of hearts as follows.

Group (A) ischaemic-reperfused hearts were reimplanted and reperfused without any drug administration to the donor or the recipient animals.

Group (B) lazaroid treated ischaemic-reperfused hearts were subjected to the same surgical procedure in the presence of the following pharmacological treatment: the aminosteroid U-74389G dissolved in 0.05N HCl was administered i.v. to the donor at a dose of 6 mgKg^{-1} 10 min prior to the explant and to the recipient (3 mgkg^{-1}) at the beginning of the reperfusion. A greater dose was given to the donor in order to reach a suitable myocardial concentration of this compound in spite of the short time elapsing between its administration and the heart explant.

Group (C) control hearts were from animals killed by cervical dislocation and were rapidly excised and examined.

The observers were not aware to which group the examined samples belonged.

Histological methods

Muscle biopsies from the left ventricle were immediately fixed in cold 4% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 at room temperature and postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4 at room temperature. The specimens were then dehydrated in a graded acetone series, passed through propylene oxide and embedded in Epon 812. Semi-thin sections, 1 to 2 μm thick, were cut and stained with toluidine blue-sodium tetraborate and observed under light microscope. Ultrathin sections were also obtained from the same specimens, stained with uranyl acetate and alkaline bismuth subnitrate, and examined under a transmission electron microscope.

Biochemical assays

After 30 min of reperfusion, the heart was freeze-clamped with aluminium tongs, immersed in liquid nitrogen, powdered in mortar and divided into two aliquots. The first aliquot was weighed and homogenized for 20 s in ice-cold 10% trichloroacetic acid (ratio 1:5, w/v) using an Ultraturrax homogenizer at half maximal speed. After 10 min of centrifugation at 6000 rpm the resultant supernatant was assayed for malondialdehyde (MDA) using the thiobarbituric acid (TBA) test[10]. Briefly, an aliquot of the supernatant was reacted with an equal volume of 0.67% (w/v) TBA in boiling water for 10 min. After cooling, the absorbance was read at 532 nm and the concentration of MDA was calculated on an ε value of 153 000. The second aliquot of pulverized tissue was weighed and homogenized for 20 s in ice-cold 0.6 N $HClO_4$ as above described. After centrifugation the supernatant was neutralized with 5 M K_2CO_3, centrifuged again and used for ATP and creatine phosphate (CP) determinations. These were performed using spectrophotometric procedures according to Jaworek and Welsch for ATP[11] and Heinz and Weißer for CP[12]. Control hearts were rapidly excised and treated as previously described. Serum creatine kinase levels were assayed using a commercially available kit (Boehringer Mannheim).

Data analysis

Data are reported as means ± SEM Statistical analysis was performed by Student's *t*-test. The minimum level of significance was set at $P<0.05$.

RESULTS

In addition to the histological and biochemical findings listed below we observed that with the reperfusion, hearts started ventricular fibrillation (V.F.) which spontaneously converted to a sinus tachycardia. This was observed in all transplanted hearts, however, there was a difference between treated and untreated animals in the duration of V.F. In fact sinus rhythm resumption occurred in the first minute in the treated and always after a longer time in the untreated rats.

Histological findings

The results of our morphological examinations may be summarized as follows.

Control myocardium (C group). The control myocardium showed normal structural and ultrastructural features. In particular, mithocondria were dense with cristae evenly distributed within the organelle. A substantial amount of glycogen granules was dispersed along the myofibrils (data not shown).

Ischaemic and reperfused myocardium (A group). Ischaemia followed by reperfusion resulted in severe myocardial injuries and endothelial alterations. By LM examination, the cardiac tissue showed a marked and diffuse oedema associated with extravasation of red blood cells. Irreversible tissue injuries could be detected with ultrastructural analysis. In fact, mitochondria showed high amplitude swellings associated with a clearing of the intramitochondrial matrix and disappearance of cristae (Fig. 1A). Besides the presence of an interfibre oedema, an intermyofibrillar oedema affected almost all the fibres, most of which also revealed clumping and margination of the nuclear chromatin. The endothelial lining of small blood vessels also appeared to be seriously damaged. Indeed, microvascular endothelial cells exhibited large areas of plasma membrane disruptions with accumulation of cellular debris nearby (Fig. 1B). Evidence of severe endothelial damage also included a bulging of the nucleus into the vessel lumen and the disappearance of endothelial intercellular junctions.

Lazaroid treated ischaemic and reperfused myocardium (B group). The treatment with lazaroids markedly attenuated most of the tissue injuries. LM examination showed that, with respect to the untreated ischaemic and reperfused samples, a less severe oedema widened the spaces among the myocardial fibers. Absence of intermyofibrillar oedema was also a prominent finding. At the ultrastructural analysis, only mild changes in the mitochondria were observed, consisting in focal areas of low amplitude swelling with some loss of definition of cristae (Fig. 1C). However, these mitochondrial alterations seemed to be compatible with a maintained functionality. The most striking structural difference between treated and untreated ischaemic/reperfused samples was found at the endothelial lining. Indeed, after treatment with lazaroids, the vascular endothelium appeared to preserve its structure (Fig. 1D). Quite normal appearing endothelial cells were joined by intact intercellular junctions.

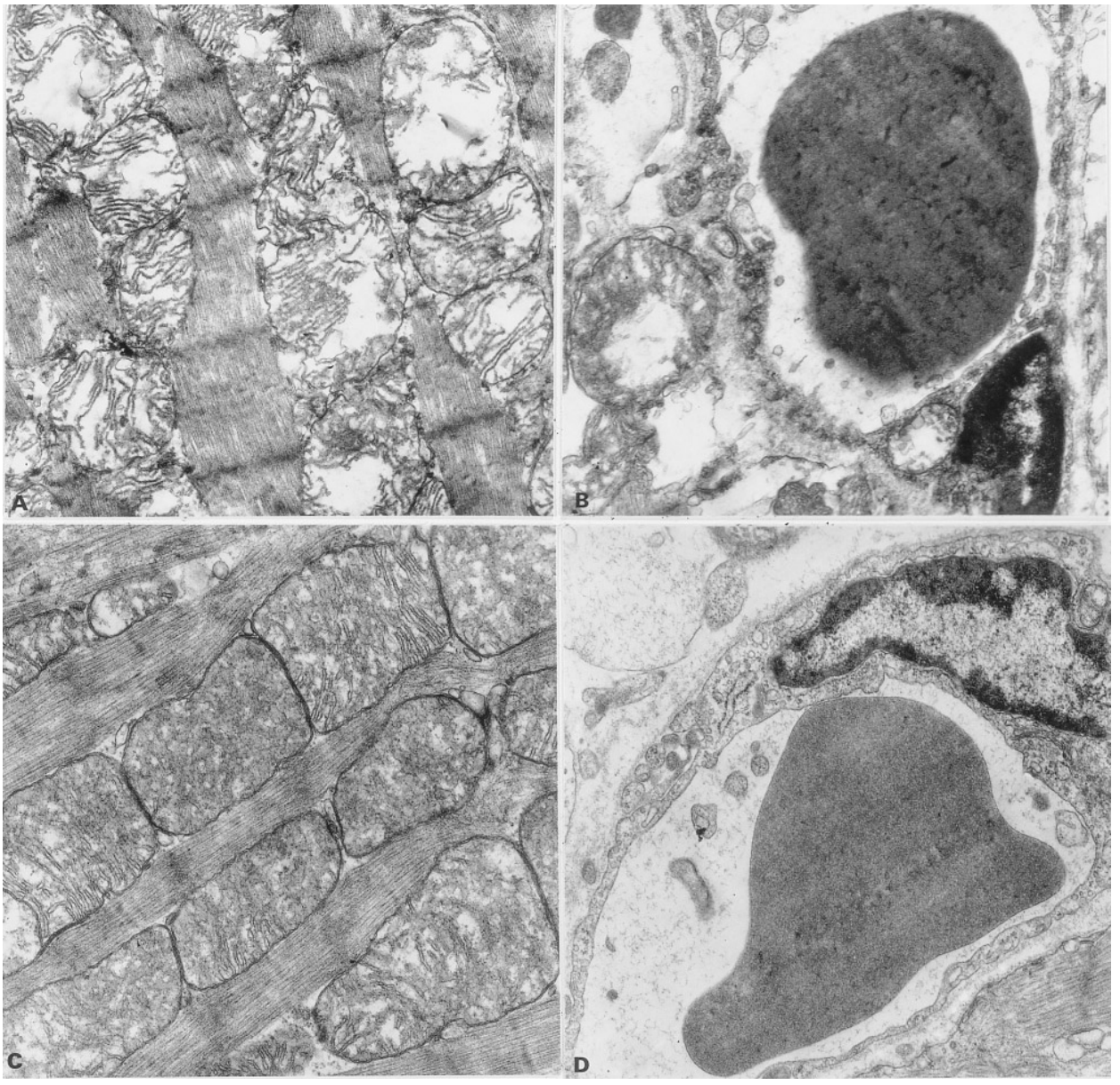

Fig. 1. (A) Ischaemic and reperfused myocardium. Mitochondria appear to be seriously injured: they lack cristae and show clearing of the intramitochondrial matrix (T.E.M×14 400). (B) Ischaemic and reperfused myocardium. Severe alterations are visible at the endothelial lining of a capillary (T.E.M.×26 400). (C) Treated ischaemic and reperfused myocardium. Mitochondria exhibit only low amplitude swellings (T.E.M.×17 600). (D) Treated ischaemic and reperfused myocardium. The endothelium appears to be completely preserved and quite similar to that of the control myocardium (T.E.M.×8 800).

Biochemical modifications

To determine possible biochemical consequences of lazaroid treatment in terms of energy metabolic status and lipoperoxidative damage ATP, CP and MDA assays were carried out in rat transplanted hearts after 30 min of reperfusion.

As summarized in Table I, in the hearts of A group (ischaemic-reperfused without pharmacological treatment) both ATP and CP concentrations were strongly and significantly reduced with respect to control hearts ($P<0.01$ for ATP and CP). In comparison with A group, the hearts of B group (ischaemic-reperfused in the presence of U74389G treatment) exhibited significantly ($P<0.01$) higher ATP and CP concentrations: these were close to those observed in the control hearts, thus reflecting a lesser depletion in high energy phosphorylated compounds.

As for the behaviour of MDA, when heart transplantation was associated with lazaroid treatment we observed a lower myocardial content of this substance compared to the levels found in hearts transplanted without treatment (Fig. 2).

Release of creatine kinase from the heart is considered one of the reliable signs of myocardial cell suffering or as a sign of an increase in cell permeability. Serum creatine kinase levels after 30 min of reperfusion were significantly lower in U-74389G treated than in untreated animals, even if both values

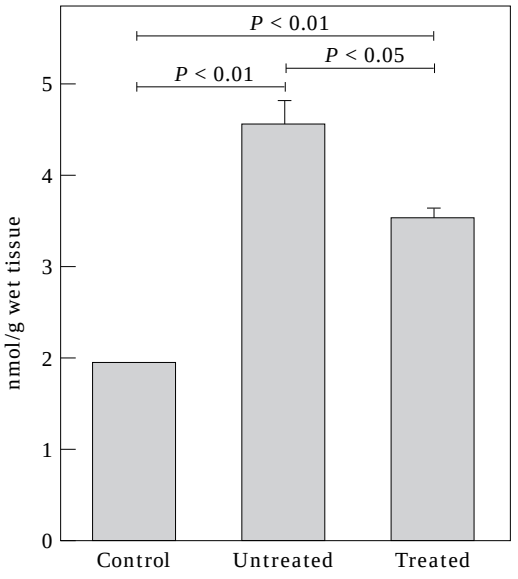

Fig. 2. Malondialdehyde content in transplanted donor hearts. Donor rat heart was isolated and immersed in saline solution at 4°C for 2 h and then transplanted and reperfused for 30 min. After reperfusion the heart was freeze-clamped, immersed in N_2 and treated as described under Materials and Methods. Control—mean ± SEM (n=5) in control hearts; untreated—mean ± SEM (n=7) in transplanted donor hearts without drug treatment; treated—mean ± SEM (n=7) in transplanted donor hearts after U-74389G treatment of donor (6 mgkg^{-1}) and recipient (3 mgkg^{-1}) animals. Statistical differences between groups are also presented.

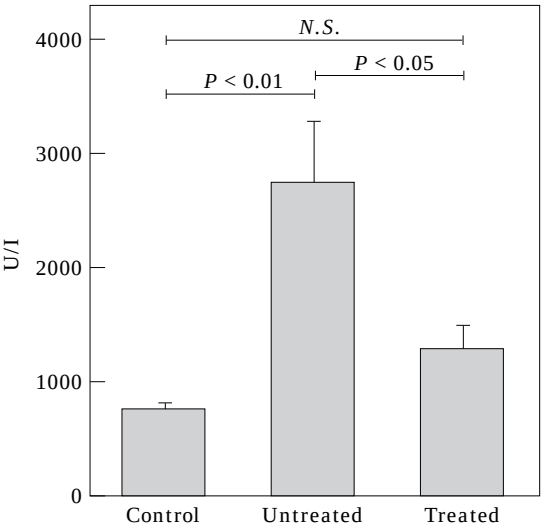

Fig. 3. Creatine kinase activity in serum of rat submitted to heterotopic cardiac transplantation. The blood was drawn from the inferior vena cava of the recipient rat submitted to cardiac transplantation after 30 min of reperfusion. Control—mean ± SEM (n=5) in serum of sham operated rats; untreated—mean ± SEM (n=7) in serum of recipient animals without drug treatment; treated—mean ± SEM (n=7) in serum of recipient animals after U-74389G treatment of donor (6 mg kg^{-1}) and recipient (3 mg kg^{-1}) animals. Statistical differences between groups are also presented.

resulted higher compared to those observed in control (sham operated) rats (Fig. 3).

DISCUSSION

Many drugs have been studied experimentally to evaluate their ability in decreasing heart damage due to ischaemia-reperfusion mechanisms, and some of them have already been entered for clinical use.

Mannitol[13] is usually added to the prime of extracorporeal circulation; catalase and superoxide dismutase have been shown to reduce myocardial depression after acute myocardial infarction[14] and deferoxamine addition to cardioplegic solution reduces post-ischaemic damage[15].

Table I
Myocardial high energy phosphates in heterotopically transplanted rat heart

Group	*ATP* (*μmol g^{-1} wet tissue*)	*CP* (*μmol g^{-1} wet tissue*)
(A) untreated	0·53 ± 0·09*	0·69 ± 0·03*
(B) treated	0·93 ± 0·06	1·13± 0·02
(C) control	1·11 ± 0·10	1·07± 0·11

Donor heart was isolated and immersed in saline solution at 4°C for 2 h, then transplanted and reperfused for 30 min. After reperfusion the heart was freeze-clamped, immersed in N_2 liquid and treated as described under Materials and Methods. (A) mean ± SEM (n=7) in transplanted hearts without drug treatment; (B) mean ± SEM (n=7) in transplanted hearts after U-74389G treatment of donor (6 mg kg^{-1}) and recipient (3 mg kg^{-1}) animals; (C) mean ± SEM (n=5) in control hearts*P<0.01 *vs* control or treated animals.

The experimental model commonly used to investigate post-ischaemic recovery of myocardial tissue is the temporary occlusion of one coronary artery and its subsequent reperfusion [14,16,17] which can mimic the mechanisms of surgical or cardiological (percutaneous transluminal coronary angioplastic) reperfusion.

The present study, aimed to investigate the efficiency of lazaroids in reducing myocardial ischaemia-reperfused injuries, was performed on hearts subjected to heterotopic transplantation which represent an unusual and demanding model. The reasons for our choice are as follows:

(1) The explanted, stored, retransplanted and reperfused heart is a model of whole ischaemia-reperfusion without the intervention of possible intercoronary anasthomoses.

(2) Functional recovery of the transplanted heart is dependent upon length of ischaemic time which, in turn, is responsible for ischaemic-reperfusion injury.

Any drug reducing this damage will improve myocardial recovery and could be added to the storage solution or to the recipient, prolonging safe ischaemic time and increasing the donor's pool.

We did not use an isolated heart preparation because this type of system may lead to myocardial oedema and tissue swelling.

Our findings clearly show that the aminosteroid U-74389G exerts a cardioprotective effect on the ultrastructural alterations of the ischaemic-reperfused myocardium. In the absence of lazaroid treatment, the endothelial lining of small blood vessels appeared seriously damaged, being formed by degenerating cells with areas of plasma membrane disruptions. These endothelial alterations may be the result of membrane lipid peroxidative processes leading to an enhanced capillary permeability[18,19] Accordingly, we observed that a marked intermyocyte oedema enlarges the spaces among the myocardial fibres. In addition, typical peroxidative injuries[20–22] occurred at the cytoplasmic and organcellular membranes within the myocardial fibre. Indeed, an intermyofibrillar oedema associated with a loss of glycogen store, swollen and disrupted mitochondria represents a common finding in the ischaemic-reperfused myocardium. On the contrary, after lazaroid treatment, only mild changes occurred at the endothelial and myocardial level. In particular, most of the endothelium lining appeared intact and well preserved, suggesting an unaltered membrane permeability. Indeed, the oedema among the myocardial fibres appears reduced with respect to that of the untreated ischaemic and reperfused heart. Mitochondria still presented minor signs of ultrastructural alterations showing only a low amplitude which widen the space among the cristae; however, these alterations, contrary to those observed in the untreated ischaemic-reperfused myocardium, seem to be totally compatible with a maintained functionality of the organelle. As for the biochemical determinations, the results are similar to those reported by Hendry *et al.*[23] who studied the effects of 21-aminosteroids on an isolated heart preparation and found that, after 4 hours of cold ischaemia, ATP and CP concentrations were significantly higher in treated than in untreated hearts.

In any case, taking together the morphological and biochemical data emerging from the present study, it seems reasonable to suppose that the effect of U-74389G in our model of ischaemic-reperfused heart mainly consists of a relevant reduction of lipoperoxidative damage, as indicated by the behaviour of myocardial MDA levels, which were significantly reduced by lazaroid treatment. Such a protective effect is evident at various levels, notably in the endothelial, sarcolemmal and organcellular membranes. Endothelial membrane protection may explain the reduction of oedema among myocardial fibres; protection of the sarcolemmal membrane against a modification in its permeability, as indicated by serum creatine kinase levels, may be a mechanism preventing adenosine leakage which, together with an improvement in mitochondrial membrane stability, could contribute to a reduced depletion of ATP and related high energy compounds.

In conclusion, our data taken together suggest that lazaroid has a beneficial effect in protecting cardiac muscle from ischaemia-reperfusion injuries, at least from those arising during a heart transplantation procedure. Work is currently in progress to study the effects and the action mechanism(s) of aminosteroids in other experimental models of the ischaemic-reperfused heart.

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